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Continuous Proteolysis of Casein by the Cell Bound Protease of *Allium Sepa*

Hamim Bokhari[a*], Muhammad Rafiq Khan[b]

[a]*Govt Post Graduate Islamia College Cooper Road Lahore.,Pakistan*

[b]*Center of Policy and Environment, Lahore School of Economics, 19-km Road, Lahore, Burki Pakistan*

[a]*Email: a1hamim@yahoo.com*

[b]*Email: mrkhan 1939@yahoo.com*

Abstract

The work reported in this paper was carried out to develop continuous proteolytic system to prepare protein hydrolysis products for different biological applications. Continuous proteolysis of casein substrate prepared in phosphate buffer (pH 7) by the seeds of *Allium sepa packed* in a column was attempted. Assuming that the seeds contained a mixture of soluble and cell-bound proteases, the soluble protease was eluted by passing buffer (pH 7) through the packed seeds. The buffered substrate was subsequently run into the column to constitute the system of continuous proteolysis. The products of proteolysis collected in the form of 5 mL fractions were assayed by Lowry's method. The system operated successfully for proteolysis on continuous basis. The data indicated that the life of cell bound protease of *Allium sepa* was 42 days.

Key Words: Protein; Continuous proteolysis; Casein; *Allium sepa*.

1. Introduction

Allium sepa (onion) carries a great importance in the field of modern medicine and thus finds extensive applications as treatment of diseases such as diabetes mellitus .

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* Corresponding author.

It contains different phytochemical compounds which are used in basic research to determine their possible properties in humans [1,2,3,4]. There are different proteases in *Allium sepa* seeds and have nutritional and functional properties [5,6].

Several methods are used for continuous hydrolysis by enzymes. Immobilization is a technique in which enzymes bind themselves with some matrices filled in chromatographic column by passing buffer through the substrate hydrolyzed products eluted out of the column. Such immobilized enzymes have increased stability [7,8].

In Pakistan work has been carried out to hydrolyze casein by proteases immobilized on activated charcoal [9] and on DEAE-cellulose [10], Amberlite CG-400[11] and promising results have been reported. The work being reported here is the extension of the work but with the change that instead of using artificially immobilized enzyme matrices, the naturally occurring proteases bound to plant seed cells reported to exhibit high protease content packed in chromatographic column were used to carry out enzyme hydrolysis on continuous basis. The first two experiments were conducted on naturally occurring cell bound protease of *Carum Copticum* [12] and *Nigella Sativa* [13] with high proteolytic activity. Continuing the same work being reported here to develop proteolytic system by the seeds of *Allium Sepa* in a chromatographic column.

1.2 Materials and methods

Allium sepa commonly known as onion seeds are easily available in the local market. It was particularly examined for the elimination of any impurity contained in it.

High quality casein supplied by Merck was used. A column of size 18 x 1 inch (dia) was used for setting up the cell-bound protease bed in it.

1.*3Assay of protease activity*: The protease activity assay was carried out by the Method of McDonald and Chen [14]. The same method was applied to determine the products of proteolysis eluted from the column. In this method, an adequate volume of the test sample usually 1mL was incubated with buffered substrate. The soluble products formed as a result of protease action were lower proteins, peptides and amino acids in the form of a mixture. Undigested proteins were precipitated with an adequate volume of 5 mL trichloroactetic acid (5%). The contents were allowed to settle down and then filtered. The protein hydrolyzed was measured by developing a blue colour with Folin-Ciocalteu phenol reagent and reading the optical density of the colour at 660 nm in a (Cecil 7200) spectrophotometer. The unit of protease activity was defined as the amount of the enzyme that caused an increase in optical density of 0.1 under the assay conditions defined.

1.4 Protease activity of the soluble enzyme

Before setting a system for continuous proteolysis, the soluble part of the enzyme present in the seeds was removed and assayed. 35 g of *Allium sepa* seeds were stirred with 350 mL of buffer pH 7. The mixture was allowed to settle for three days for maximum dissolution of soluble enzyme in the buffer and subsequent removal. The mixture was then filtered. The filtrate was assayed for protease activity using 1 mL sample. 1 mL

of the filtrate was incubated with 4 mL of buffered casein (1 %) pH 7 for 1 h at 30 °C. The undigested soluble protein was precipitated with 5 mL of trichloroactetic acid (5 %) that also stopped the reaction. The mixture was allowed to stand for 0.5 h and then filtered through Whatmann filter paper no. 41. 1 mL of the filtrate was transferred to 5 mL of alkaline mixture prepared by mixing 100 mL of sodium carbonate (2 %), 1 mL sodium potassium tartrate (2.7 %) and 1 mL copper sulfate (1 %). Then 2 mL of NaOH (1 N) was added to make the mixture alkaline. After at least 10 min, 0.5 mL Folin-Ciocaulteu phenol reagent was added and then contents mixed. The blue colour produced was read at 660 nm in a (Cecil 7200) spectrophotometer. Blank was prepared by the same procedure except 1 mL sample was substituted by 1 mL of buffer (pH 7).

1.5 Protease activity of the powdered Allium sepa

The dry seeds of *Allium sepa* were powered using electric grinder. The protease activity of the seed powder was determined to have an idea about the total units of protease present in the seed sample. 100 mg of seed powder along with 1 mL buffer (pH 7) constituted the sample and heat denatured 100 mg powder made the blank. The remaining procedure was the same as above.

1.6 Packing of the column

Suspension of the seeds was prepared in buffer pH 7 as above and transferred to the column. The column was allowed to stand for 2 h for the proper settling of the cells of *Allium sepa.* The tap was opened to allow the buffer to flow. The flow of the drops was adjusted so that each drop fell after 30 to 40 s. It took 0.5 h to collect a 5 mL fraction. The soluble enzyme was collected eluting with buffer as 5 mL fractions. The elution was carried till the buffer coming out of the column had no protease activity. This also guaranteed that the soluble enzyme had been completely removed. To keep the flow continuous, 20 mL buffer was transferred taking care that the seed bed was not disturbed. It took about three days to remove the soluble enzyme completely. Ten fractions were collected and their protease activity was assayed. The optical density was plotted against fraction number to construct the elution diagram.

1.7 Continuous proteolysis of casein

The process of continuous proteolysis of casein was started after the soluble enzyme had washed out of the column. For this purpose, 1% casein substrate was run through the column and 5 mL of fractions of the enzyme-affected substrate were collected as above. Thus, casein had reasonable time to be in contact with the cell-bound protease of *Allium sepa.* Every fraction was analyzed for the products of proteolysis. For analysis, 2.5 mL of every alternate fraction was treated with 2.5 mL of trichloroacetic acid (5 %) to precipitate unhydro-lyzed protein. The precipitate was filtered off and 1 mL of the filtrate was used to develop colour with Folin and Ciocalteu phenol reagent as was done in assay of protease activity. The colour was read at 660 nm in the spectrophotmeter. The processes were continued for 25 d and for first 26 days 2.5 mL of fractions were collected daily. The column was run for 7 h a day and for 6 d a week. After 26 d, the running was stopped for 1 week to seek whether the protease under goes a change without flow of the substrate. This means that determination of extent of proteolysis was resumed on 34th day.

2. Results

Table1: Protease activity of powered *Allium sepa*

Medicinal seed powder	Protease activity units/100mg	Protease activity units/g	Protease activity units in 35g sample
Allium sepa	3.8	3.8	1330

Table2.2: Protease activity of *Allium sepa* Seeds

Medicinal seed	Soluble protease activity units/ml	Protease activity in total extract volume 350ml
Allium sepa	2.4	840

2.1.Elution of soluble protease of Allium sepa

The change in protease activity per mL in terms of OD of different fractions analyzed with the number of the fractions collected is exhibited in Fig. 1

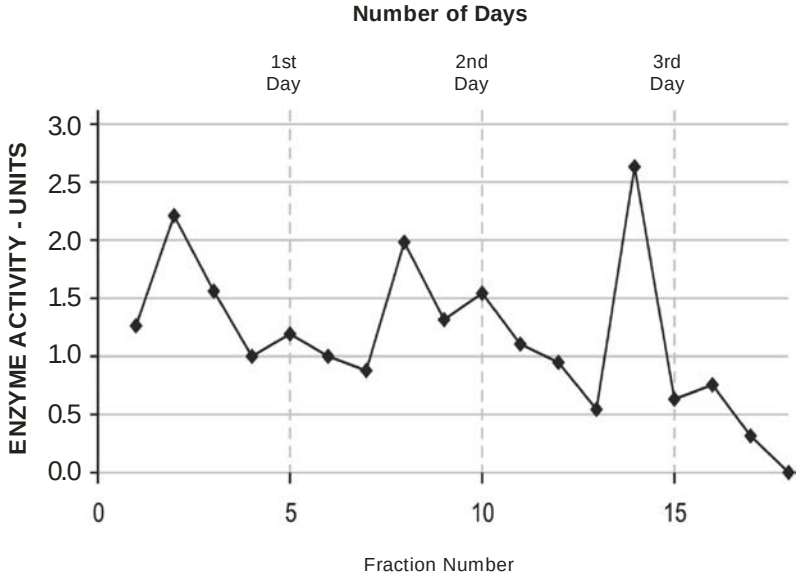

Figure 1: Change in enzyme activity of the soluble protease from the seeds of *Allium sepa* packed in the column during elution with 0.2 M citrate phosphate buffer pH 7.0 (Days1-3).

The profile indicates that the enzyme activity of soluble protease decreases exhibiting rise and fall with the addition of fresh 0.2M citrate phosphate buffer pH 7.0 and finally become zero with the addition of fresh 0.2M Citrate phosphate buffer pH 7.0.Different peaks are encountered in this profile.

2.2. Hydrolysis of Casein by cell bound protease of Allium sepa seeds

The progress of proteolysis of casein by the cell bound protease of *Allium sepa* is demonstrated in Fig. 2 for the first 26 d. The results indicate that the rate of hydrolysis of casein by the cell bound 'protease went on increasing with the increase in fraction number or elution volume if interpreted in terms of peaks encountered in the pattern. The rate of hydrolysis thus increased with the passage of time.

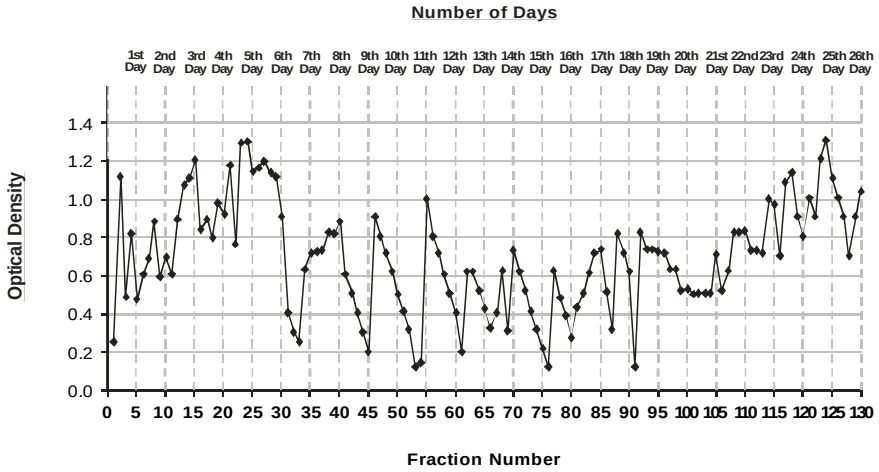

Figure 2: Elution diagram showing the extent of proteolysis by immobilized enzyme after the application of casein substrate in 0.2 M citrate phosphate buffer at pH 7.0 to *Allium sepa* seeds column (Days 1-26)

The progress of proteolysis of casein after seven days stoppage is shown below

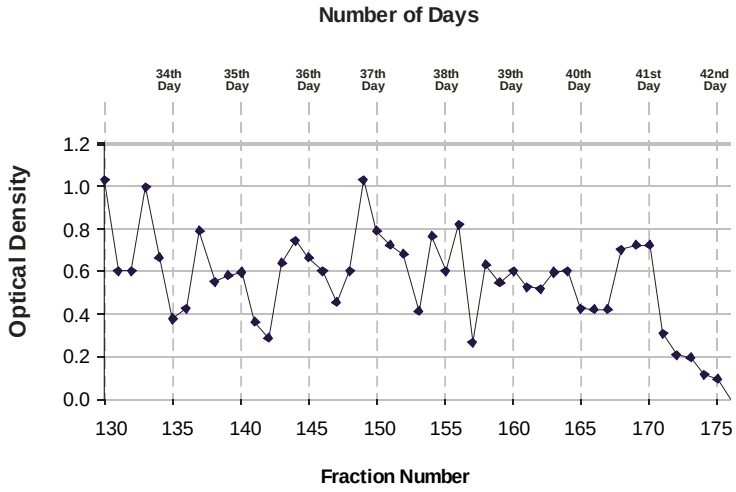

Figure 3: Elution diagram showing the extent of proteolysis by immobilized enzyme after keeping the *Allium sepa* seeds column closed for one-week continuous elution (Days 34-42).

The results indicate the life span of column packed with *Allium sepa* seems to be 42 days.

3. Discussion

The protease activity of the bulked soluble enzyme was 2.4 units / mL. As the total volume of buffer added was 350 mL, the total number of activity units present in 350 mL was 840. In other words, the units of activity of soluble protease per gram were 24. The number of protease units per 100 mg, were 3.8. Thus, unit/g and unit/35 g came out to be 38 and 1330, respectively. This means that the seed powder contained protease more than that solubilized by the buffer pH 7 from the whole seeds and the balance was naturally immobilized

Different peaks are encountered in the profile of soluble protease of *Allium sepa* seeds (Fig.1) whose number is more than those encountered in soluble protease profile of *Carum copticum*[12] This means that the number of soluble proteases present in the soluble enzyme of *Allium sepa* is greater than that present in soluble enzyme of *Carum copticum* [12] Elution diagram of *Nigella sativa*[13] seeds shows that it has one prominent peak. Thus it contains only one major protease component.

Soluble protease activity of natural seeds is compared with the soluble enzyme activity of *Carica papaya* eluted from artificial matrices[9,10,11] the average peak heights were similar. Of course, the protease activity of first fraction of every seed is different from other seeds. The highest was of *Carum copticum* [12]while the lowest was of *Nigella sativa*. [13].This may be due to the reason that *Carum copticum*[12] seeds are softer than those of *Nigella sativa* [13] and *Allium sepa*, which are quite compact. This means that more enzymes is solublized out of *Carum copticum* [12] during the three day suspension in buffer and thus more it remains sticking to its seeds in the column. Thus initially more enzyme is washed out as explained earlier but in case of other seeds which have similar texture less enzyme remains sticking and thus less elutes out of the column. So, the protease activity of the first fraction will be naturally higher in case of *Carum copticum* [12] After the column is washed out with buffer, the activity drop in case of *Carum copticum* [12] is due to the fact that the protease was almost completely solublized out of the seeds in the suspension transferred to the column for packing while in case of other, not all the protease was washed out and much remained in the seeds and was solublized out with passage of time during the running of the column as indicated by an increase and then decrease in protease activity (Fig. 1)The height of peaks of different fractions of *Allium sepa* is quite similar to *Nigella sativa*[13] It means that their cell bound proteases show similar behavior.

The progress of proteolysis of casein by cell bound protease of *Allium sepa*(Fig2)compared with *Nigella sativa*[13] which indicates more sharp peaks were obtained than *Allium sepa*.It means that *Nigella sativa*[13] has more proteolytic activity in neural medium as compared to *Allium sepa* .The progress of proteolysis of casein after 7days stoppage(Fig 3) compared with *Nigella sativa* [13] which indicates The extent of proteolysis was significantly high, which went on increasing with the passage of time. The data indicated that the life of cell bound protease of *Allium sepa* was 42 days. Thus developed system being based on natural seeds can be better applied for the proteolysis of proteins for making amino acids and lower protein products which can be used in drips as instantaneous source of energy and nutrition.

4. Conclusion

The extent of proteolysis was significantly high, which went on increasing with the passage of time. The data indicated that the life of cell bound protease of Allium sepa was 42 days. Thus the developed system, being based on harmless natural seeds of Allium sepa, can be better applied for the proteolysis of some proteins for making soluble amino acid preparations such as drips for instantaneous supply of nitrogen to the patients needing nutrition for their survival

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